Operating windows for transaminase processes using thermodynamic and biocatalyst constraints

Tufvesson, Pär; Lima Ramos, Joana; Jensen, Jacob Skibsted; Woodley, John

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Enzymatic transesterification of egg-yolk phosphatidylcholine with castor oil

Witold Gladkowski, Anna Chojnacka, Czesław Wawrzeńczyk
Department of Chemistry, Wroclaw University of Environmental and Life Sciences, 50-375 Wroclaw, Poland
E-mail: glado@poczta.fm

The introduction of hydroxy acids into the natural phospholipids is the subject of increasing research interest. Hydroxylated phospholipids have useful surface properties, higher moisture retention and increased water dispersibility therefore they can find the application in food industry as the emulsifying agents or in bakery as staling retardants [1]. They are also used in cosmetic and pharmaceutical industry as the source of biologically active hydroxyacids.
The most common hydroxy fatty acid is ricinoleic acid which occurs in castor seeds (*Ricinus communis*). Few chemical and enzymatic methods have been developed so far to produce phospholipids containing ricinoleic acid [2-4]. Enzymatic approach have advantages: mild reaction conditions and regio-and stereospecificity of the biocatalysts.
In this communication we report the results of direct incorporation of ricinoleic acid to the lecithin isolated from egg yolk using direct transesterification with castor oil as the natural acyl donor. We applied two enzyme preparations: lipase from Candida antarctica (Novozym 435) and lipase from *Thermomyces lanuginosa* (Lipozym TL IM). The effect of various conditions (enzyme dose, temperature, castor oil:lecithin molar ratio, water activity - a_w) on the incorporation of ricinoleic acid was studied. The highest content of ricinoleic acid in modified lecithin (31%) was achieved in following conditions: temperature 50oC, enzyme dose 30% (w/w of total substrates), molar ratio castor oil:lecithin 5:1, aw=0.22. Detailed results of the enzymatic reactions will be presented.

Acknowledgements:
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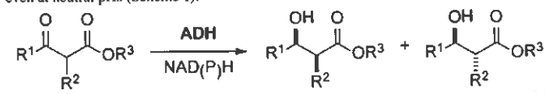
Obtaining of β-Hydroxyesters Through Dynamic Processes Catalysed by Alcohol Dehydrogenases

Iván Lavandera[a], Aníbal Cuetos[a], Ana Rioz-Martínez[a], Fabricio R. Bisogno[a], Barbara Mautner[b], Cristina Rodríguez[a], Gonzalo de Gonzalo[a], Wolfgang Kroutil[b], Vicente Gotor[a]
[a]Organic and Inorganic Department, Instituto de Biotecnología de Asturias, University of Oviedo, C/ Julián Clavería 8, 33006 Oviedo, Spain; [b]Department of Chemistry, Organic and Bioorganic Chemistry, University of Graz, Heinrichstrasse 28, 8010 Graz, Austria
E-mail: lavanderaivan@uniovi.es

In the last few years, the employment and development of dynamic protocols in order to obtain enantio- or diastereomerically pure compounds starting from easily available racemic starting material has largely increased. In this sense, more and more processes involving enzymatic and homogeneous catalysis in dynamic conditions are designed to achieve successful transformations to synthesise optically pure high-added value derivatives.[1]
One of the most typical examples of a dynamic process is the reduction of α-substituted β-ketoesters (Scheme 1) to obtain the corresponding alcohols with high diastereomeric excesses (*des*).[2] This process has been successfully achieved using both metal- and enzyme catalysis due to high acidity of the α-hydrogen that ensures a fast substrate racemisation even at neutral pHs (Scheme 1).



Scheme 1. Bioreduction of α-substituted β-ketoesters affording a mixture of diastereomeric alcohols employing ADHs.

Concerning the bioreduction of these and related derivatives, the first examples were shown employing whole cells such as baker's yeast, and although in some cases excellent conversions and enantiomeric excesses (*ees*) were achieved, the presence of several active enzymes also depleted the selectivity in many cases.[2] More recently, the development of these dynamic protocols using purified or overexpressed alcohol dehydrogenases (ADHs),[3] has overcome this problem although in hand with efficient techniques to recycle the expensive cofactor needed in these processes. Herein we would like to show the use of several purified and overexpressed ADHs applied to the bioreduction of several α-substituted β-ketoesters in order to obtain the corresponding alcohols with excellent enantiomeric and diastereomeric excesses. Obviously, depending on the enzyme and the substrate structure, different results were observed and they will be discussed to gain a deeper insight into the different possibilities that these biocatalysts can provide.

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Rhamnulose-1-Phosphate aldolase from *Thermotoga maritima* as a new biocatalyst for the synthesis of nitrocyclitols

Isabel Oroz-Guinea[a], Flora Camps-Bres[b,c], Christine Guérard-Hélaine[b,c], Israel Sánchez-Moreno[a], Eduardo García-Junceda[a] and Marielle Lemaire[b,c]**
[a]Departamento de Química Bio-Orgánica, Instituto de Química Orgánica General, CSIC. 28006 Madrid, Spain.
[b]Laboratoire SEESIB, Clermont Université, Université Blaise Pascal, 24 avenue des Landais, F-63177 Aubière.
[c]CNRS, UMR 6504, 24 avenue des Landais, F-63177 Aubière.
E-mail: isabel.oroz@iqog.csic.es

Recently, Prof. Marielle Lemaire's group has developed a new methodology to access to nitrocyclitols and aminocyclitols analogs of valiolamine, validamine and valienamine which have a high potential activity as molecular chaperons for glycosidases1. The key step in this chemo-enzymatic synthesis is the aldol reaction between dihydroxyacetone phosphate (DHAP) and an aldehyde catalyzed by a DHAP-dependent aldolase. A highly stereoselective intramolecular cyclization (Henry reaction) takes place during the aldolase-catalyzed reaction and is then followed by a phosphatase-catalyzed hydrolysis step. Enantiomerically pure nitrocyclitols with four new stereocenters for each aldehyde tested were obtained in one pot.

We are involved now in extending this strategy using the rhamnulose-1-phosphate aldolase (Rham-1PA) from *Thermotoga maritima*. This enzyme has been previously cloned and characterized in our group and, as a hyperthermophilic enzyme, offers intriguing possibilities for practical catalysis like high stability under extreme (temperature, presence of organic co-solvents, etc.) and storage conditions. In this communication we describe the synthetic application of rhamnulose-1-phosphate aldolase from *Thermotoga maritima* as a new biocatalyst for the synthesis of nitrocyclitols according to the strategy described above.

Acknowledgements: We thank the Spanish Ministerio de Ciencia e Innovación (Grant CTQ2010-15418) and Comunidad de Madrid (Grant S2009/PPQ-1752) for financial support. I. Oroz-Guinea is a JAEPredoc fellow from CSIC.

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Via enzyme toolboxes to chiral diols

Justyna Kulig[a], Robert Simon[b], Wolfgang Kroutil[b], Frank Hollmann[c], Wolfgang Wiechert[a], Martina Pohl[a], Dörte Rother[a]
aInstitute of Bio- and Geosciences 1: Biotechnology, Forschungszentrum Jülich GmbH, 52425 Jülich, Germany; bDepartment of Chemistry, Karl-Franzens-Universität Graz, Heinrichstrasse 28, 8100 Graz, Austria; cDepartment of Biotechnology, Delft University of Technology, Julianalaan 136, 2628 BL Delft, The Netherlands
E-mail: j.kulig@fz-juelich.de

Chiral diols find versatile application as synthons for chemical catalysts, agrochemicals and pharmaceuticals.[1,2] The concept of combining two toolboxes of enzymes opens the access to diversely substituted enantiocomplementary vicinal diols (Fig 1).

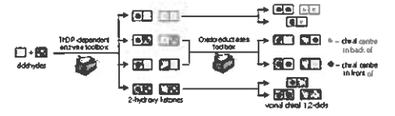

Figure 1. Combining ThDP-dependent enzymes and oxidoreductases toolboxes

First, the carboligation of cheap aldehydes by thiamine diphosphate (ThDP)-dependent enzymes yielding 2-hydroxy ketones with high chemo- and stereoselectivity takes place. [3] Subsequently the 2-hydroxy ketones are further reduced *via* NAD(P)H-dependent oxidoreductases (Fig 2).

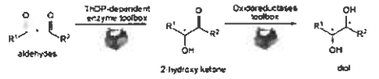

Figure 2. Stereoselective 2-step enzymatic synthesis of chiral 1,2-diols

The main focus is the stereoselective reduction of especially bulky-bulky substrates.[4,5] Promising reactions are optimised via reaction engineering and an appropriate process for the enzymatic 2-step synthesis with cofactor regeneration will be evaluated.

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Chemistry and synthetic biology in symbiosis for the synthesis of benzylisoquinoline alkaloids

Thomas Pesnot,[1] Markus C. Gershater,[1] John M. Ward[2] and Helen C. Hailes[1]
[1]Department of Chemistry, University College London, 20 Gordon Street, London
[2]Structural and Molecular Biology, University College London, Gower Street, London
t.pesnot@ucl.ac.uk

In nature, benzylisoquinoline alkaloids (BIAs) are produced by plants as a defence mechanism against herbivores or pathogens. Many BIA natural products were also identified as biologically active and are now prescribed for the treatment of a wide array of diseases including HIV, cancer and microbial infections as well as for the treatment of pain.[1] Thus, BIAs provide excellent scaffolds for the development of new compounds with enhanced biological activities. The chemical synthesis of BIAs is however hindered by many challenges including their optical activity, high polarity and multiple functionalities.[2] In order to address these issues and readily synthesise libraries of novel BIAs, we investigated the concerted use of synthetic biology and chemical synthesis.

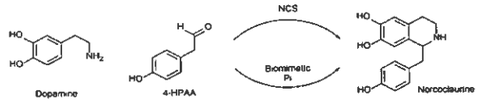

Figure: Enzymatic and biomimetic syntheses of norcoclaurine

Biosynthetically, the first committed step to BIAs is catalysed by norcoclaurine synthase (NCS) which couples dopamine with 4-hydroxyphenylacetaldehyde (4-HPAA) to yield (*S*)-norcoclaurine (Figure).[3] During our studies, we carried out expressions of native NCSs in BL21 E. Coli and measured their respective enantioselectivities. The substrate tolerance of selected NCSs was screened using our newly developed fluorescence-based assay. The assay highlighted substrate features which are essential for NCS activity and helped in understanding its catalytic mechanism. Hits from the assay were scaled up and enantiomeric excesses measured. Biomimetic reactions conditions were also developed and utilised for the rapid synthesis of naturally occurring as well as non-natural BIAs.[4] In combining chemical synthesis with synthetic biology, a wide variety of BIAs pharmaceutical candidates were produced.

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Enzymatic Carboxylation of Aromatic Compounds

Christiane Wuensch[a], Silvia M. Glueck[a] and Kurt Faber[b]
[a]Austrian Centre of Industrial Biotechnology, c/o [b]Department of Chemistry, University of Graz, Heinrichstrasse 28, A-8010 Graz, Austria
christiane.wuensch@acib.at

Due to the increasing abundance of CO_2 as a major greenhouse gas, the development of CO_2-fixation reactions for the production of chemicals is one of the main challenges in synthetic organic chemistry [1]. The Kolbe-Schmitt reaction [2], which is one of the major industrial processes for the production of aromatic carboxylic acids, requires harsh reaction conditions (high pressure and temperature). In contrast, the use of (de)carboxylases represents a 'green' alternative method for the regioselective carboxylation of aromatic compounds.
To test the viability of this novel enzymatic process various aromatic substrates were subjected to several benzoic acid (de)carboxylases in bicarbonate buffer. Among the seven tested enzymes the decarboxylases from *Aspergillus oryzae* [3], *Rhizobium sp.* [4] and *Trichosporon moniliiforme* [5] were found to be highly active. Depending on the enzyme and substrate, respectively, the carboxylated product was formed in up to 42% conversion.

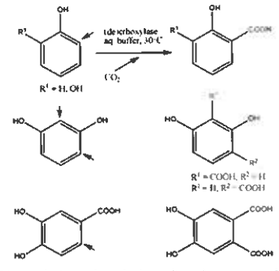

Figure 1. Biocatalytic carboxylation of various aromatic substrates.

Acknowledgemets: Financial support by the FGG is gratefully acknowledged.

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A chemoenzymatic process for the synthesis of acyclic nucleoside analogues involving the use of rabbit muscle aldolase (RAMA)

Martín A. Palazzolo[a], Adolfo M. Iribarren[a,b], Elizabeth S. Lewkowicz[a]
[a]Laboratorio de Biocatálisis y Biotransformaciones, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Roque Sáenz Peña 352, Bernal (CP 1876), Argentina; [b]Laboratorio de Química de Ácidos Nucleicos, INGEBI-CONICET, Vuelta de Obligado 2490, Ciudad Autónoma de Buenos Aires (CP 1428), Argentina
E-mail: mpalazzolo@unq.edu.ar

For more than 40 years, nucleoside analogues have shown to be a valuable source of antiviral and antitumor agents [1]. In particular, acyclic nucleosides received great interest –since the discovery of acyclovir in 1978- due to their extraordinary chemical and biological stability.
Unlike natural nucleosides, the high flexibility of the C-C linkages enables the acyclic analogues to adopt conformations that favor the formation of complexes with the enzymes involved in their metabolism [2]. Often, the absolute configuration of the stereogenic centers plays an important role in determining both their biological activity and selectivity [3]. Aldolases are enzymes that catalyse reversible, stereospecific C-C bond condensation reactions. In general, they possess strong dependence on the donor compound but show low specificity towards the acceptor substrate [4]. Owing to these facts, they are a very interesting tool for the synthesis of a variety of chiral compounds.
In this work, we describe a chemoenzymatic process for the preparation of thymidine and adenosine acyclic analogues (Figure 1). This strategy involved the suitable N-alkylation of the nucleosidic bases to generate the acceptors for the subsequent aldol condensation reaction biocatalysed by rabbit muscle aldolase (RAMA). The expected acyclic nucleoside analogues were obtained in moderate to high yields.

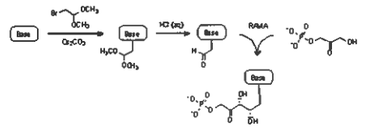

Figure 1. Chemoenzymatic synthesis of acyclic nucleoside analogues.

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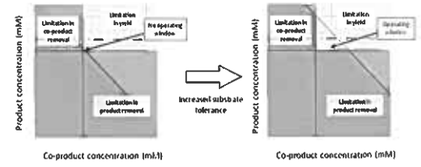
Operating windows for transaminase processes using thermodynamic and biocatalyst constraints

Pär Tufvesson, Joana Lima-Ramos, Jacob S. Jensen and John M Woodley
Center for Process Engineering and Technology, Department of Chemical and Biochemical Engineering, Technical University of Denmark (DTU), DK-2800 Lyngby, Denmark
E-mail: pt@kt.dtu.dk

Transaminase catalysed reactions have been demonstrated as an attractive alternative to conventional resolution strategies for chiral amines, both in the laboratory and to a much more limited extent in industrial processes [1]. Indeed, one challenge that needs to be overcome when developing such process for industrial application is how to achieve high yields in the face of an unfavorable reaction equilibrium. Several different solutions have been suggested to overcome this, such as evaporation of a volatile co-product or the in-situ enzymatic degradation of the co-product, e.g. pyruvate.

This work describes a methodology to analyse the feasibility of a process by combining a set of physical and biological constraints to create an operating window. For instance the equilibrium constant of the reaction determines the concentration of the product and co-product in solution that must be maintained in order to meet a target reaction yield [2]. Additionally, the performance of the biocatalyst (including inhibition) gives another set of constraints.

The methodology is demonstrated for the development of a biocatalytic transamination reaction and shows how it can be used to guide the selection of technologies and configurations, as well as identification and establishment of development targets.



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